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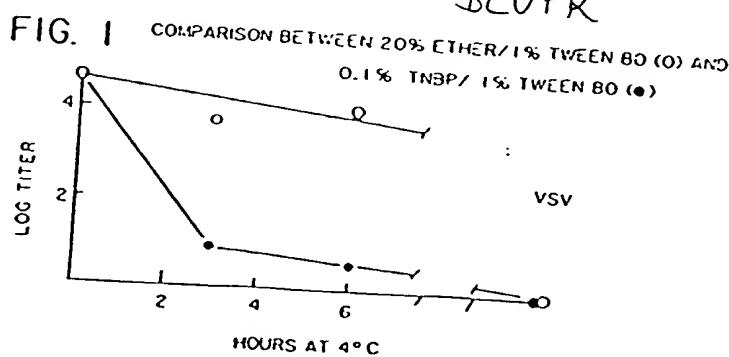
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㉓ Undenatured virus-free biologically active protein derivatives.

㉔ A mammalian blood protein-containing composition such as whole blood, plasma, serum, plasma concentrate, cryoprecipitate, cryosupernatant, plasma fractionation precipitate or plasma fractionation supernatant substantially free of hepatitis and other lipid coated viruses with the yield of protein activity to total protein being at least 80% is disclosed. The protein-containing composition is contacted with di- or trialkylphosphate, preferably a mixture of trialkylphosphate and detergent, usually followed by removal of the di- or trialk-

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PROTEIN  
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Field of the Invention

This invention relates to undenatured virus-free biologically active protein-containing compositions. More especially, this invention relates to the inactivation of viruses, especially lipid coated viruses, e.g., hepatitis B in human blood, blood component, blood plasma or any fraction, concentrate or derivative thereof containing blood proteins or non-blood sources including normal or cancer cells, the exudate from cancer or normal cells grown in culture, hybridomas, in products from gene splicing (DNA), etc., by the use of di- or trialkyl phosphates, and to the resultant products. In particular, this invention relates to blood plasma or other plasma protein-containing compositions which are to be rendered substantially free of hepatitis B and/or non-A and non-B hepatitis or other viral infectivity, such blood plasma or fractions thereof having valuable labile proteins, such as, for example, factor VIII.

DISCUSSION OF PRIOR ART

Numerous attempts have been made to inactivate viruses such as hepatitis B virus (HBV) in mammalian, especially human, blood plasma. It is the practice in some countries to effect inactivation of the hepatitis B virus in the blood plasma by contacting the plasma with a viral inactivating agent of the type which crosslinks with the proteinaceous portion of hepatitis B virus, or which interacts with the nucleic acid of the virus. For instance, it is known to attempt to inactivate hepatitis B virus by contact with an ~~aldehyde~~ such as formaldehyde whereby crosslinking to the protein is effected and the hepatitis B

1       virus is inactivated. It is also known to effect 2  
2       inactivation of the virus by contact with beta-propiolactone  
3       (BPL), an agent which acts on the nucleic acid of the virus.  
4       It is further known to use ultraviolet (UV) light,  
5       especially after a beta-propiolactone treatment.

6              Unfortunately, these agents often alter, denature  
7       or destroy valuable protein components especially so-called  
8       stable blood coagulation factors of the plasma under condi-  
9       tions required for effective inactivation of virus infectivity.  
10         For instance, in such inactivation procedures, factor VIII is  
11       inactivated or denatured to the extent of 50-90% or more of  
12       the factor VIII present in the untreated plasma. Because of  
13       the denaturing effects of these virus inactivating agents,  
14       it is necessary in the preparation of derivatives for admin-  
15       istration to patients to concentrate large quantities of plasma  
16       so that the material to be administered to the patient once  
17       again has a sufficient concentration of the undenatured  
18       protein for effective therapeutic treatment. This concentra-  
19       tion, however, does not affect reduction of the amount of de-  
20       natured protein. As a result, the patient not only receives  
21       the undenatured protein but a quantity of denatured protein  
22       often many times that of the undenatured protein.  
23

24              For instance, in the inactivation of hepatitis B  
25       virus in human blood plasma by beta-propiolactone, there is  
26       obtained as a result thereof, a plasma whose factor VIII has  
27       been 75% inactivated. The remaining 25% of the factor VIII  
28       is therefore present in such a small concentration, as a  
29       function of the plasma itself, that it is necessary to  
30       concentrate large quantities of the factor VIII to provide

1 sufficient concentration to be of therapeutic value. Since  
2 such separation techniques do not efficiently remove  
3 denatured factor VIII from undenatured factor VIII, the  
4 material administered to the patient may contain more  
5 denatured protein than undenatured protein. Obviously, such  
6 inactivation is valuable from a standpoint of diminishing  
7 the risk of hepatitis virus infection. However, it requires  
8 the processing of large quantities of plasma and represents  
9 significant loss of valuable protein components. Furthermore,  
10 administration of large amounts of denatured proteins may ren-  
11 der these antigenic to the host and thus give rise to autoim-  
12 mune diseases, or perhaps, rheumatoid arthritis.

13  
14 The loss of these valuable protein components is  
15 not limited to factor VIII, one of the most labile of the val-  
16 uable proteins in mammalian blood plasma. Similar protein  
17 denaturation is experienced in respect of the following  
18 other valuable plasma components: coagulation factors II,  
19 VII, IX, X; plasmin, fibrinogen (factor 1) IgM, hemoglobin,  
20 interferon, etc.

21 Factor VIII, however, is denatured to a larger  
22 extent than many of the other valuable proteins present in  
23 blood plasma.

24 As a result of the foregoing, except in the  
25 processing of serum albumin, a stable plasma protein  
26 solution which can withstand pasteurization, it is largely  
27 the practice in the United States in respect of the  
28 processing of blood proteins to take no step in respect of  
29 the sterilization for inactivation of viruses. As a result,  
30 recipients of factor VIII, gamma-globulin, factor IX,

1 fibrinogen, etc., must accept the risk that the valuable  
2 protein components being administered may be contaminated  
3 with hepatitis viruses as well as other infectious viruses.  
4 As a result, these recipients face the danger of becoming  
5 infected by these viruses and having to endure the damage  
6 which the virus causes to the liver and other organ systems  
7 and consequent incapacitation and illness, which may lead to  
8 death.

*B-Lipopolymer*

9 The BPL/UV inactivation procedure discussed above  
10 has not so far been adopted in the United States for  
11 numerous reasons, one of which lies in the fact that many  
12 researchers believe that BPL is itself deleterious since it  
13 cannot be removed completely following the inactivation and  
14 thus may remain in plasma and plasma derivatives. BPL has been  
15 shown to be carcinogenic in animals and is dangerous even to  
16 personnel handling it.

17 Other methods for the inactivation of hepatitis B  
18 virus in the plasma are known, but are usually impractical.  
19 One method involves the addition of antibodies to the plasma  
20 whereby an immune complex is formed. The expense of  
21 antibody formation and purification add significantly to the  
22 cost of the plasma production; furthermore, there is no  
23 assurance that a sufficient quantity of hepatitis B or  
24 non-A, non-B virus is inactivated. There is currently no  
25 test for non-A, non-B antibodies (although there is a test  
26 for the virus); hence, it is not possible to select plasma  
27 containing high titers of anti non-A, non-B antibody.

28 It is to be understood that the problems of  
29 inactivation of the viruses in plasma are distinct from the  
30 problems of inactivation of the viruses themselves due to

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the copresence of the desirable proteinaceous components of the plasma. Thus, while it is known how to inactivate the hepatitis B virus, crosslinking agents, for example, glutaraldehyde, nucleic acid reacting chemicals, for example BPL or formaldehyde, or oxidizing agents, for example chlorox, etc., it has been believed that these methods are not suitable for the inactivation of the virus in plasma due to the observation that most of these activating agents (sodium hypochlorite, formaldehyde, beta-propiolactone) denatured the valuable proteinaceous components of the plasma.

United States Patent 4,315,919 to Shanbrom describes a method of depyrogenating a proteinaceous biological or pharmaceutical product by contacting such proteinaceous product with a non-denaturing amphiphile.

United States Patent 4,314,997 to Shanbrom describes a method of reducing pyrogenicity, hepatitis infectivity and clotting activation of a plasma protein product by contacting the product with a non-denatured amphiphile.

Both Shanbrom '919 and '997 contemplate the use of a non-ionic detergent, for example, "Tween 80" as the amphiphile. It will be shown hereinafter that treatment with "Tween 80" by itself is relatively ineffective as a viral inactivating agent.

United States Patent 3,962,421 describes a method for the disruption of infectious lipid-containing viruses for preparing sub-unit vaccines by contacting the virus in an aqueous medium with a wetting agent and a trialkylphosphate. Such aqueous medium is defined as allantonic fluid, tissue culture fluid, aqueous extract or suspension of central nervous

1 system tissue; blood cell eluate and an aqueous extract of  
2 suspension of fowl embryo. The patent does not describe  
3 hepatitis, nor is it concerned with preparation of blood de-  
4 rivatives containing labile blood protein substantially free of  
5 viral infectivity. It is only concerned with disrupting the  
6 envelope of lipid containing viruses for the production of  
7 vaccines and not with avoiding or reducing protein denaturation  
8 en route to a blood derivative.

9 Problems may also exist in deriving valuable pro-  
0 teins from non-blood sources. These sources include, but are  
1 not limited to, mammalian milk, ascitic fluid, saliva, placental  
2 extracts, tissue culture cell lines and their extracts includ-  
3 ing transformed cells, and products of fermentation. For in-  
4 stance, the human lymphoblastoid cells have been isolated which  
5 produce alpha interferon. However, the cell line in commercial  
6 use today contains Epstein-Barr virus genes. It has been a  
7 major concern that the use of interferon produced by these  
8 cells would transmit viral infection or induce viral caused  
9 cancerous growth.

0 The present invention is directed to achieving  
1 three goals, namely, (1) a safe, (2) viral inactivated  
2 protein-containing composition, (3), without incurring substan-  
3 tial protein denaturation. As shown above, these three goals  
4 are not necessarily compatible since, for example, beta-  
5 propiolactone inactivates viral infectivity, but is unsafe and  
6 substances such as formaldehyde inactivate viruses; but also  
7 substantially denature the valuable plasma proteins, for ex-  
8 ample, factor VIII.

9 It, therefore, became desirable to provide a proc-  
0 ess for obtaining protein-containing compositions which does

1 not substantially denature the valuable protein components  
2 therein and which does not entail the use of a proven carcinogenic agent. More especially, it is desirable to provide blood  
3 protein-containing compositions in which substantially all of  
4 the hepatitis viruses and other viruses present are inactivated  
5 and in which denatured protein such as factor VIII account for only a small amount of the total amount of these  
6 proteins in the blood protein-containing composition.

7  
8  
9 It is a further object to provide products from  
10 cancer or normal cells or from fermentation processes following  
11 gene insertion which are substantially free of virus, especially lipid-containing viruses.  
12  
13

14 SUMMARY OF THE INVENTION

15 It has now been discovered, quite surprisingly,  
16 that while most of the viral inactivating agents denature  
17 factor VIII and other valuable blood plasma proteins, that  
18 not all viral inactivating agents have such effect. It has  
19 been discovered that a protein-containing composition such  
20 as whole blood, blood cell proteins, blood plasma, a blood  
21 plasma fractionation precipitate, a blood plasma fractionation  
22 supernatant, cryoprecipitate, cryosupernatant, or portion  
23 or derivative thereof or serum or a non-blood product  
24 produced from normal or cancerous cells (e.g. via recombinant  
25 DNA technology) is contacted for a sufficient period of time  
26 with a dialkylphosphate or a trialkylphosphate that lipid containing  
27 viruses such as the hepatitis viruses present in the composition  
28 are virtually entirely inactivated without substantial  
29 denaturation of proteins therein. By contacting blood protein  
30

1 mixture or concentrate thereof or fraction thereof with a di-  
2 or trialkylphosphate, followed by removal of the di- or trial-  
3 kylphosphate, hepatitis viruses can be substantially inacti-  
4 vated, e.g., to an inactivation of greater than 4 logs, while  
5 realizing a yield of protein activity to total protein of  
6 at least 80%.

7 By such procedures there is provided a blood  
8 protein-containing composition such as mammalian whole blood,  
9 blood cell derivatives (e.g., hemoglobin, alpha-interferon,  
10 T-cell growth factor, platelet-derived growth factor, etc.),  
11 plasminogen activator, blood plasma, blood plasma fraction,  
12 blood plasma precipitate (e.g., cryoprecipitate, ethanol pre-  
13 cipitate or polyethylene glycol precipitate), or supernatant  
14 (e.g., cryosupernatant, ethanol supernatant or polyethylene  
15 glycol supernatant), characterized by the presence of one or  
16 more blood proteins such as labile blood factor VIII having  
17 a total yield of protein activity to total protein of at least  
18 80%, preferably at least 85%, more preferably 95% and most  
19 preferably 98% to 100%, said blood protein-containing composi-  
20 tion having greatly reduced or virtually no hepatitis viruses.  
21 Virus in a serum is determined by infectivity titrations.  
22

23 By the inactivation procedure of the invention,  
24 most if not virtually all of the hepatitis viruses contained  
25 therein would be inactivated. The method for determining  
26 infectivity levels by in vivo chimpanzees is discussed by  
27 Prince, A.M., Stephen, W., Brotman, B. and van den Ende, H.C.,  
28 "Evaluation of the Effect of Beta-propiolactone/Ultraviolet  
29 Irradiation (BPL/UV) Treatment of Source Plasma on Hepatitis  
30 Transmission by factor IV Complex in Chimpanzees, Thrombosis  
and Haemostasis", 44: 138-142, 1980.

The hepatitis virus is inactivated by treatment

1 with the di- or trialkylphosphate described herein, and is not  
2 inactivated because of inclusion in the plasma of antibodies  
3 which bind with the hepatitis viruses and form immune complexes

4 Inactivation of virus is obtained to the extent of  
5 at least "4 logs", i.e., virus in a serum is totally inacti-  
6 vated to the extent determined by infectivity studies where that  
7 virus is present in the untreated serum in such a concentration  
8 that even after dilution to  $10^4$ , viral activity can be meas-  
9 ured.

10

11 BRIEF DESCRIPTION OF THE DRAWINGS

12 Fig. 1 shows virus inactivation as a function of  
13 log titer value versus time for VSV virus (vesicular stomatitis  
14 virus) treated according to the present invention and treated  
15 with ether/Tween 80. The lower log titer for treatment ac-  
16 cording to the present invention indicates greater virus inac-  
17 tivation;

18 Fig. 2 shows virus inactivation as a function of  
19 log titer value versus time for Sindbis virus treated accord-  
20 ing to the present invention and treated with ether/Tween 80;

21 Fig. 3 shows virus inactivation as a function of  
22 log titer value versus time for Sendai virus treated according  
23 to the present invention and treated with ether/Tween 80;

24 Fig. 4 shows virus inactivation as a function of  
25 log titer value versus time for EMC virus (a non-lipid  
26 coated virus) treated according to the present invention and  
27 treated with ether/Tween 80; 4°C

28 Fig. 5 is a plot of log titer value versus hours  
29 for VSV virus for TNBP/Tween 80 at 0°C and at room temperature  
30 and TNBP alone (at room temperature);

1 Fig. 6 is a plot of log titer value versus hours  
2 for Sindbis virus for TNBP/Tween 80 at 0°C and at room  
3 temperature and TNBP alone (at room temperature);

4 Fig. 7 is a plot of log titer value versus hours  
5 for Sendai virus for TNBP /Tween 80 at 0°C and at room  
6 temperature and TNBP alone (at room temperature); and

7 Fig. 8 is a plot of log titer value versus hours  
8 for EMC virus for TNBP/Tween 80 at 0°C and at room tempera-  
9 ture and TNBP alone (at room temperature).

10 The Sindbis, Sendai and VSV viruses are typical  
11 lipid containing viruses and are used herein to determine the  
12 effect of di- or trialkylphosphate on lipid coated viruses  
13 generally.

14

15 DETAILED DESCRIPTION OF THE INVENTION

16 Blood is made up of solids (cells, i.e.,  
17 erythrocytes, leucocytes, and thrombocytes) and liquid  
18 (plasma). The cells contain potentially valuable substances  
19 such as hemoglobin, and they can be induced to make other  
20 potentially valuable substances such as interferons, growth  
21 factors, and other biological response modifiers. The plasma  
22 is composed mainly of water, salts, lipids and proteins. The  
23 proteins are divided into groups called fibrinogens, serum  
24 globulins and serum albumins. Typical antibodies (immune globu-  
25 lins) found in human blood plasma include those directed  
26 against infectious hepatitis, influenza H, etc.

27 Blood transfusions are used to treat anemia  
28 resulting from disease or hemorrhage, shock resulting from  
29 loss of plasma proteins or loss of circulating volume.

1 diseases where an adequate level of plasma protein is not  
2 maintained, for example, hemophilia, and to bestow passive  
3 immunization.

4 Whole blood must be carefully typed and cross  
5 matched prior to administration. Plasma, however, does not  
6 require prior testing. For certain applications, only a  
7 proper fraction of the plasma is required, such as factor  
8 VIII for treatment of hemophilia or von Willebrand's disease.

9 With certain diseases one or several of the  
10 components of blood may be lacking. Thus the administration  
11 of the proper fraction will suffice, and the other components  
12 will not be "wasted" on the patient; the other fractions can  
13 be used for another patient. The separation of blood into  
14 components and their subsequent fractionation allows the pro-  
15 teins to be concentrated, thus permitting concentrates to be  
16 treated. Of great importance, too, is the fact that the  
17 plasma fractions can be stored for much longer periods than  
18 whole blood and they can be distributed in the liquid, the  
19 frozen, or the dried state. Finally, it allows salvaging from  
20 blood banks the plasma portions of outdated whole blood that  
21 are unsafe for administration as whole blood.  
22

23 Proteins found in human plasma include prealbumin,  
24 retinol-binding protein, albumin, alpha-globulins, beta-  
25 globulins, gamma-globulins (immune serum globulins), the  
26 coagulation proteins (antithrombin III, prothrombin, plasmino-  
27 gen, antihemophilic factor-factor VIII, fibrin-stabilizing  
28 factor-factor XIII, fibrinogen), immunoglobins(immunoglobulins  
29 G, A, M, D, and E), and the complement components. There are  
30 currently more than 100 plasma proteins that have been de-

scribed<sup>12</sup>. A comprehensive listing can be found in "The Plasma Proteins", ed. Putnam, F.W., Academic Press, New York (1975).

Proteins found in the blood cell fraction include hemoglobin, fibronectin, fibrinogen, enzymes of carbohydrate and protein metabolism, etc. In addition, the synthesis of other proteins can be induced, such as interferons and growth factors.

A comprehensive list of inducible leukocyte proteins can be found in Stanley Cohen, Edgar Pick, J.J. Oppenheim, "Biology of the Lymphokines", Academic Press, N.Y. (1979).

Blood plasma fractionation generally involves the use of organic solvents such as ethanol, ether and polyethylene glycol at low temperatures and at controlled pH values to effect precipitation of a particular fraction containing one or more plasma proteins. The resultant supernatant can itself then be precipitated and so on until the desired degree of fractionation is attained. More recently, separations are based on chromatographic processes. An excellent survey of blood fractionation appears in Kirk-Othmer's Encyclopedia of Chemical Technology, Third Edition, Interscience Publishers, Volume 4, pages 25 to 62, the entire contents of which are incorporated by reference herein.

The major components of a cold ethanol fractionation are as follows:

<u>Fraction</u>	<u>Proteins</u>
I	fibrinogen; cold insoluble globulin; factor VIII:properdin
II and III	IgG; IgM; IgA; fibrinogen; beta-lipoprotein; prothrombin; plasminogen; plasmin inhibitor; factor V; factor VII; factor IX; factor X; thrombin; antithrombin; isoagglutinins; cer-

	<u>Fraction</u>	<u>-13-</u>	<u>Proteins</u>	
1				0131740
2			utoplasmin; complement C'1, C'3	
3	IV-1		alpha <sub>1</sub> -lipoprotein, cerutoplasmin; plasmin- inhibitor; factor IX; peptidase; alpha-and- beta-globulins	
4				
5				
6	IV-4		transferrin; thyroxine binding globulin; serum esterase; alpha <sub>1</sub> -lipoprotein; albumin; alkaline phosphatase	
7				
8	V		albumin; alpha-globulin	
9				
10	VI		alpha <sub>1</sub> -acid glycoprotein; albumin	
11				

The above fractionation scheme can serve as a basis for further fractionations. Fraction II and III, for example, can be further fractionated to obtain immune serum globulin (ISG).

Another fractionation scheme involves use of frozen plasma which is thawed into a cryoprecipitate containing AHF (antihemophilic factor) and fibronectin and a cryosupernatant. The cryoprecipitate is then fractionated into fibronectin and AHF.

Polyethylene glycol has been used to prepare high purity AHF and non-aggregated ISG.

High risk products with respect to the transmission of hepatitis B and non-A, non-B are fibrinogen, AHF and prothrombin complex, and all other blood protein preparations except immune serum globulin and, because they are pasteurized, albumin solutions. Hepatitis tests presently available can indicate the presence of hepatitis B surface antigen, but there is presently no screening test for non-A, non-B hepatitis.

The present invention is directed to contacting

1 with di- or trialkylphosphate a blood protein-containing  
2 composition such as whole mammalian blood, blood cells  
3 thereof, blood cell proteins, blood plasma thereof,  
4 precipitate from any fractionation of such plasma,  
5 supernatant from any fractionation of such plasma, cryo-  
6 precipitate, cryosupernatant or any portions or derivatives  
7 of the above that contain blood proteins such as, for example,  
8 prothrombin complex (factors II, VII, IX and X) and  
9 cryoprecipitate (factors I and VIII). The present invention  
10 is also concerned with contacting di- or trialkylphosphate  
11 with a serum containing one or more blood proteins.  
12 Furthermore, the present invention is directed to contacting  
13 di- or trialkylphosphate with a blood protein-containing  
14 fraction containing at least one blood protein such as the  
15 following: factor II, factor VII, factor VIII, factor IX,  
16 factor X, fibrinogen and IgM. Additionally, the present  
17 invention concerns contacting a cell lysate or proteins induced  
18 in blood cells with di- or trialkylphosphate.

19 Such blood protein-containing composition is con-  
20 tacted with a dialkylphosphate or a trialkylphosphate  
21 having alkyl groups which contain 1 to 10 carbon atoms, espe-  
22 cially 2 to 10 carbon atoms. Illustrative members of trial-  
23 kylphosphates for use in the present invention include tri-  
24 (n-butyl) phosphate, tri-(t-butyl) phosphate, tri-  
25 (n-hexyl) phosphate, tri-(2-ethylhexyl) phosphate, tri-  
26 (n-decyl) phosphate, just to name a few. An especially  
27 preferred trialkylphosphate is tri-(n-butyl) phosphate.  
28 Mixtures of different trialkylphosphates can also be  
29 employed as well as phosphates having alkyl groups of  
30

different alkyl chains, for example, ethyl, di(n-butyl) phosphate. Similarly, the respective dialkylphosphates can be employed including those of different alkyl group mixtures of dialkylphosphate. Furthermore, mixtures of di- and trialkyl phosphates can be employed.

Di- or trialkylphosphates for use in the present invention are employed in an amount between about 0.01 mg/ml and about 100 mg/ml, and preferably between about 0.1 mg/ml and about 10 mg/ml.

The di- or trialkylphosphate can be used with or without the addition of wetting agents. It is preferred, however, to use di- or trialkylphosphate in conjunction with a wetting agent. Such wetting agent can be added either before, simultaneously with or after the di- or trialkylphosphate contacts the blood protein-containing composition. The function of the wetting agent is to enhance the contact of the virus in the blood protein-containing composition with the di- or trialkylphosphate. The wetting agent alone does not adequately inactivate the virus.

Preferred wetting agents are non-toxic detergents. Contemplated nonionic detergents include those which disperse at the prevailing temperature at least 0.1% by weight of the fat in an aqueous solution containing the same when 1 gram detergent per 100 ml of solution is introduced therein. In particular there is contemplated detergents which include polyoxyethylene derivatives of fatty acids, partial esters of sorbitol anhydrides, for example, those products known commercially as "Tween 80", "Tween 20" and polysorbate 80" and nonionic oil soluble water detergents such as that sold commercially under the

1 trademark "Triton X 100" (oxyethylated alkylphenol). Also  
2 contemplated is sodium deoxycholate as well as the "Zwitter-  
3 gents" which are synthetic zwitterionic detergents known as  
4 "sulfobetaines" such as N-dodecyl-N, N-dimethyl-2-ammonio-1  
5 ethane sulphonate and its congeners or nonionic detergents  
6 such as octyl-beta-D-glucopyranoside.

7 Substances which might enhance the effectiveness  
8 of alkylphosphates include reducing agents such as mercapto-  
9 ethanol, dithiothreitol, dithioerythritol, and dithiooctanoic  
10 acid. Suitable nonionic surfactants are oxyethylated alkyl  
11 phenols, polyoxyethylene sorbitan fatty acid esters, poly-  
12 oxyethylene acids, polyoxyethylene alcohols, polyoxyethylene  
13 oils and polyoxyethylene oxypropylene fatty acids. Some spe-  
14 cific examples are the following:

15 alkylphenoxyethoxy (30) ethanol  
16 polyoxyethylene (2) sorbitan monolaurate  
17 polyoxyethylene (20) sorbitan monopalmitate  
18 polyoxyethylene (20) sorbitan monostearate  
19 polyoxyethylene (20) sorbitan tristearate  
20 polyoxyethylene (20) sorbitan monooleate  
21 polyoxyethylene (20) sorbitan trioleate  
22 polyoxyethylene (20) palmitate  
23 polyoxyethylene (20) lauryl ether  
24 polyoxyethylene (20) cetyl ether  
25 polyoxyethylene (20) stearyl ether  
26 polyoxyethylene (20) oleyl ether  
27 polyoxyethylene (25) hydrogenated castor oil  
28 polyoxyethylene (25) oxypropylene monostearate

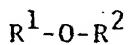
29 The amount of wetting agent, if employed, is not  
30 crucial, for example, from about 0.001% to about 10%.  
31 preferably about 0.01 to 1.5% can be used.

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1 Di- and trialkylphosphates may be used in  
2 conjunction with other inactivating agents such as alcohol  
3 or ethers with or without the copresence of wetting agents  
4 in accordance with copending application Serial No. 368,250  
5 entitled "Sterilized Plasma and Plasma Derivatives and Process  
6 Therefor", assigned to the assignee hereof.

7 The ether or alcohol can be added in an amount  
8 of 1 to 50%, preferably 5 to 25% by weight, based on the volume  
9 of blood plasma, or concentrate or other blood plasma protein-  
10 containing composition to be treated.

11 Particularly contemplated ethers for inactivation  
12 use in accordance with the invention are those having the  
13 formula



14 wherein

15  $R^1$  and  $R^2$  are independently  $C_1-C_{18}$  alkyl or  
16 alkenyl which can contain an O or S atom in the chain,  
17 preferably  $C_1-C_8$  alkyl or alkenyl. Especially contemplated  
18 ethers are dimethyl ether, diethyl ether, ethyl propyl  
19 ether, methyl-butyl ether, methyl isopropyl ether and  
20 methyl isobutyl ether.

21 Alcohols contemplated include those of the formula



22 wherein

23  $R^3$  is a  $C_1$  to  $C_{18}$  alkyl or alkenyl radical which  
24 can contain one or more oxygen or sulfur atoms in the chain  
25 and which can be substituted by one or more hydroxyl groups.

26 Especially contemplated alcohols are those where  
27 the alkyl or alkenyl group is between 1 and 8 atoms.  
28

-18-

Particularly contemplated alcohols include methanol. **0131740**  
ethanol, propanol, isopropanol, n-butanol, isobutanol,  
n-pentanol and the isopentanols. Also contemplated are  
compounds such as ethylene glycol, 1,2-propylene glycol,  
1,3-propane diol, 1,4-butanediol, 2-hydroxy isobutanol  
(2-methyl, 1,2-dihydroxypropane).

Treatment of blood protein-containing compositions  
with trialkylphosphate is effected at a temperature between  
-5°C and 70°, preferably between 0°C and 60°C. The time of  
such treatment (contact) is for at least 1 minute, preferably  
at least 1 hour and generally 4 to 24 hours. The treatment  
is normally effective at atmospheric pressure, although  
subatmospheric and superatmospheric pressures can also  
be employed.

Normally, after the treatment, the trialkylphosphate and other inactivating agents, for example, ether, are removed, although such is not necessary in all instances, depending upon the nature of the virus inactivating agents and the intended further processing of the blood plasma protein-containing composition.

To remove ether from plasma the plasma is generally subjected to a temperature of 4°C to 37°C with a slight vacuum imposed to draw off residual ether. Preferably means are provided to spread the plasma as a thin film to insure maximum contact and removal of the ether. Other methods for removal of ether in activating agents include:

- (1) bubbling of nitrogen gas;
- (2) diafiltration using ether insoluble, e.g. "TEFLON", microporous membranes

- 1 which retain the plasma proteins;
- 2 (3) absorption of desired plasma components
- 3 on chromatographic or affinity
- 4 chromatographic supports;
- 5 (4) precipitation, for example, by salting
- 6 out of plasma proteins;
- 7 (5) lyophilization, etc.

8 When alcohol or nonionic detergents are employed  
9 with the trialkylphosphate they are removed by (2) to (5)  
10 above.

11 Di- or trialkylphosphate can be removed as  
12 follows:

- 13 (a) Removal from AHF can be effected by  
14 precipitation of AHF with 2.2 molal glycine  
15 and 2.0 M sodium chloride
- 16 (b) Removal from fibronectin can be effected by  
17 binding the fibronectin on a column of  
18 insolubilized gelatin and washing the bound  
19 fibronectin free of reagent.

20 Generally speaking, any ether present is initially  
21 removed prior to removal of any detergent. The ether may be  
22 recovered for reuse by the use of suitable distillation/  
23 condenser systems well known to the art.

24 Alcohol is normally removed together with  
25 detergent. If the detergent includes both alcohol and  
26 ether, the ether is normally removed before the alcohol.

27 The process of the invention can be combined with,  
28 still other modes of inactivating viruses including those for  
29 non-lipid coated viruses. For instance, a heating step can  
30 be effected in the presence of a protein stabilizer, e.g.,

1 an agent which stabilizes the labile protein (AHF) Q13st740  
2 inactivation by heat. Moreover, the heating can be carried  
3 out using stabilizers which also tend to protect all  
4 protein, including components of the virus, against heat if  
5 the heating is carried out for a sufficient length of time,  
6 e.g., at least 5 hours and preferably at least 10 hours at a  
7 temperature of 50 - 70°C, especially 60°C. By such mode the  
8 virus is preferentially inactivated, nevertheless, while the  
9 protein retains a substantial amount, e.g., ≥ 80% of its  
10 protein activity. Of course, the best treatment can also be  
11 carried out simultaneously with the alkyl phosphate treatment.

12       The treatment of plasma or its concentrates,  
13 fractions or derivatives in accordance with the present  
14 invention can be effected using di- or trialkylphosphate  
15 immobilized on a solid substrate. The same can be fixed to  
16 a macro-molecular structure such as one of the type used as  
17 a backbone for ion exchange reactions, thereby permitting  
18 easy removal of the trialkylphosphate from the plasma or  
19 plasma concentrate. Alternatively the phosphate can be  
20 insolubilized and immobilized on a solid support such as  
21 glass beads, etc., using silane or siloxane coupling agents.  
22

23       The method of the present invention permits the  
24 pooling of human blood plasma and the treatment of the  
25 pooled human blood plasma in the form of such pooled plasma.  
26 It also permits the realization of blood product derivatives  
27 such as factor VIII, gamma globulin, factor IX or the  
28 prothrombin complex (factors II, VII, IX, X), fibrinogen and  
29 any other blood derivative including HBSAg used for the  
30 preparation of HBV vaccine, all of which contain little or  
no residual infective hepatitis or other viruses.

The present invention is directed, inter alia, to producing a blood plasma protein-containing composition such as blood, blood plasma, blood plasma fractions, etc., which is substantially free of infectious virus, yet which contains a substantial amount of viable (undenatured) protein. More particularly, the present invention is directed to inactivation of lipid-containing virus and preferentially inactivation of hepatitis B and non-B, non-A virus. Other viruses inactivated by the present invention include, for example, cytomegaloviruses, Epstein Barr viruses, lactic dehydrogenase viruses, herpes group viruses, rhabdoviruses, leukoviruses, myxoviruses, alphaviruses, Arboviruses (group B), paramyxoviruses, arenaviruses, and coronaviruses.

According to the present invention, there is contemplated a protein-containing composition - a product produced from normal or cancerous cells or by normal or cancerous cells (e.g., via recombinant DNA technology), such as mammalian blood, blood plasma, blood plasma fractions, precipitates from blood fractionation and supernatants from blood fractionation having an extent of inactivation of virus greater than 4 logs of virus such as hepatitis B and non-A, non-B, and having a yield of protein activity to total protein of at least 80%, preferably at least 95% and most preferably 98% to 100%.

Further contemplated by the present invention is a composition containing factor VIII which is substantially free of hepatitis virus to the extent of having an inactivation of greater than 4 logs of the virus and a yield of protein activity to total protein of at least 80%, preferably at least 85%, more preferably at least 95% and most preferably

The process of the present invention has been described in terms of treatment of plasma, plasma fractions, plasma concentrates or components thereof. The process, however, is also useful in treating the solid components of blood, lysates or proteins secreted by cells. Thus, also contemplated are treatment of platelet concentrates, white cell (leukocyte) concentrates, and leukocyte-poor packed red cells as well as platelet rich plasma, platelet concentrates and platelet poor plasma including packed cell masses comprising the white buffy coat consisting of white blood cells above packed red cells. Also contemplated is the treatment of masses containing concentrates of granulocytes, monocytes, interferon, and transfer factor.

One can treat plasma itself according to the present invention or fresh frozen plasma, thawed frozen plasma, cryoprecipitate, cryosupernatants or concentrates from frozen plasma as well as dilution products thereof.

By the same manipulative steps discussed above, virus present in products of normal or cancerous cells can be inactivated while retaining labile protein activity in such products. For instance, by the same di- or trialkyl-phosphate treatment one can inactivate products produced using normal or cancer cells, the exudate from normal or cancerous cells, hybridomas and products produced by gene splicing. Such treatment does not substantially adversely affect the desired protein. Cells used for production of desired protein can, of course, be mammalian as well as non-mammalian cells.

Factor VIII and factor IX coagulant activities are assayed by determining the degree of correction in APTT

1 - time of factor VIII - and factor IX - deficient plasma 0131740  
2 respectively. J.G. Lenahan, Phillips and Phillips, Clin.  
3 Chem., Vol. 12, page 269 (1966).

4 The activity of proteins which are enzymes is  
5 determined by measuring their enzymatic activity. Factor  
6 IX's activity can be measured by that technique.  
7

8 Binding proteins can have their activities  
9 measured by determining their kinetics and affinity of  
10 binding to their natural substrates.

11 Lymphokine activity is measured biologically in  
12 cell systems, typically by assaying their biological  
13 activity in cell cultures.

14 Protein activity generally is determined by the  
15 known and standard modes for determining the activity of the  
16 protein or type of protein involved.

17 In order to more fully illustrate the nature of  
18 the invention and the manner of practicing the same, the  
19 following non-limiting examples are presented:

20 Example 1

21 AHF solutions were incubated with 0.1% TNBP plus  
22 1% Tween 80 for 18 hours at 4°C. These solutions were  
23 initially contacted with VSV virus, Sindbis virus and Sendai  
24 virus and thereafter brought in contact with an aqueous  
25 solution containing 0.1 weight percent of tri(n-butyl)-  
26 phosphate (TNBP) and 1.0 weight percent detergent (Tween  
27 80), with the following resultant virus inactivations:  
28 4.7 logs of vesicular stomatitis virus (VSV), 5.8 logs of  
29 Sindbis virus, and 5.0 logs of Sendai virus. The virus  
30 was added just prior to the addition of the TNBP-Tween 80.  
The yield of AHF (labile protein/total protein) was found to

1 be 86%.

2 - 24 -

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Controls in which TNBP and Tween 80 were omitted  
showed little if any viral inactivation.

The results for Example 1 are shown below in

Table I:

Table I

Temperature	Time (Hrs)	AHF U/ml	% Yld	Log Titer VSV	Virus Sindbis	Virus Sendai
4°C	Start	10.4	(100)	4.7	5.8	5.0
	3	-	-	0.9	-0.4	2.2
	6	-	-	0.6	-0.5	1.5
	18	8.9	86	-0.5	0.5	-0.5

In Fig. 1, Fig. 2, and Fig. 3, the results of Example 1 are plotted and compared to virus inactivation with ether (20%)/Tween 80 (1%). It is seen that for VSV (Fig. 1), Sindbis (Fig. 2) and Sendai (Fig. 3), inactivation was greater (lower log titer value) for treatment according to the present invention (with TNBP) than with ether/Tween 80 treatment.

In Table II, the effect of a "Tween 80" alone in the inactivation of viruses is shown. The data shows that little if any inactivation is due to "Tween 80".

TABLE II

EFFECT OF TWEEN 80 (1%) ALONE ON VIRUS INACTIVATION

Experiment	Temperature (°C)	Duration (Hrs)	Inactivation (log#)			EMC
			VSV	Sindbis	Sendai	
1	0°C	3	0.3	0.0	0.0	0.4
2	0°C	18	ND*	-0.1	0.7	0.5
	22°C	18	ND*	-0.1	-0.3	0.0

# log titer control minus log titer treated

\* not done

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1    Example 2

2                  Example 1 was repeated, but at 22°C. The results  
3    for Example 2 are summarized below in Table III:

5    Temperature	6    Time (Hrs)	7    Table III			8    Log Titer Virus
		9    AIIF	10    V/ml	11    %Yld	
12    22°C	13    Untreated	14    8.3	15    (100)	16    4.4	17    5.1
18	19    3	20    8.2	21    99	22    <-0.4	23    <-0.5
24	25	26	27	28	29
30	31	32	33	34	35

11                 The present invention may be embodied in other  
12   specific forms without departing from the spirit or  
13   essential attributes thereof and, accordingly, reference  
14   should be had to the appended claims, rather than to the  
15   foregoing specification, as indicating the scope of the  
16   invention.

2      -26-

3      1. A process for obtaining a protein-containing  
4      composition which is substantially free of lipid-containing  
5      viruses without incurring substantial protein denaturation  
6      comprising contacting said protein-containing composition  
7      with an effective amount of di- or trialkylphosphate for a  
sufficient period of time.

8

9      2. A process according to claim 1 wherein di- or  
10     trialkylphosphate has alkyl groups which contain 1 to 10  
11     carbon atoms.

12

13     3. A process according to claim 2 wherein said  
14     trialkylphosphate has alkyl groups which contain 2 to 10  
15     carbon atoms.

16

17     4. A process according to claim 2 wherein said  
18     trialkylphosphate is tri-n-butyl phosphate.

19

20     5. A process according to claim 1 wherein said  
21     contacting is conducted in the presence of a wetting agent.

22

23     6. A process according to claim 5 wherein said  
24     wetting agent is a non-ionic detergent.

25

26     7. A process according to claim 5 wherein said  
27     wetting agent is added to said protein-containing  
28     composition prior to contacting said protein-containing  
29     composition with said di- or trialkylphosphate.

1  
2        8. A process according to claim 5 wherein said  
wetting agent is added simultaneously with said di- or  
trialkylphosphate to said protein-containing composition.

3  
4  
5        9. A process according to claim 5 wherein said  
wetting agent is added after said di- or trialkylphosphate  
contacts said protein-containing composition.

6  
7        10. A process according to claim 6 wherein said  
detergent is a partial ester of sorbitol anhydrides.

8  
9        11. A process according to claim 1 further  
comprising conducting said contacting in the presence of an  
inactivating agent selected from the group consisting of  
ethers and alcohols.

10  
11      12. A process according to claim 5 further  
comprising conducting said contacting in the presence of an  
inactivating agent selected from the group consisting of  
ethers and alcohols.

12  
13      13. A process according to claim 1 wherein said  
protein-containing composition is selected from the group  
consisting of whole blood, blood plasma, a plasma  
concentrate, a precipitate from any fractionation of such  
plasma, a supernatant from any fractionation of said plasma,  
a serum, a cryoprecipitate, a cell lysate, and proteins  
induced in blood cells.

1        14. A process according to claim 1 wherein said  
2        blood protein-containing composition contains one or more  
3        proteins selected from the group consisting of fibrinogen,  
4        factor II, factor VII, factor VIII, factor IX, factor X,  
5        factor I, immunoglobins, prealbumin, retinol-binding  
6        protein, albumin, alpha-globulins, beta-globulins, gamma-  
7        globulins, factor III and the complement components,  
8        fibronectin, antithrombin III, hemoglobin, interferon,  
9        T-cell growth factor, plasminogen activator.

10        15. A process according to claim 1 wherein said  
12        protein-containing composition is the product of a non-blood  
13        normal or cancerous cell or the product of gene splicing.

14        16. A process according to claim 1 wherein  
15        following said contacting with said di- or trialkylphosphate,  
16        said di- or trialkylphosphate is removed.

19        17. A process according to claim 1 wherein said  
20        period of time is between about 1 minute and about 30 hours.

22        18. A process according to claim 1 wherein said  
23        contacting is conducted at a temperature of between about  
24        0°C and about 70°C.

26        19. A process according to claim 1 wherein said  
27        di- or trialkylphosphate is present in an amount between  
28        about 0.001% and about 1%.

1                   33. A process according to claim 24 wherein said  
2 composition is substantially free of infective lipid-containing  
3 viruses.

4  
5                   34. A product of a non-blood normal or cancerous  
6 cell comprising an active protein and inactivated virus  
7 wherein the amount of active protein is at least 80% of the  
8 total protein.  
9  
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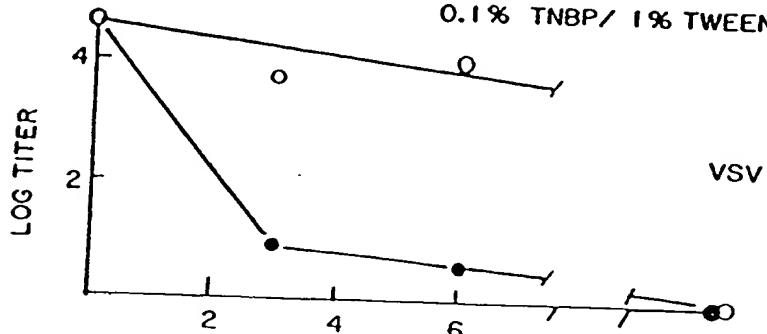
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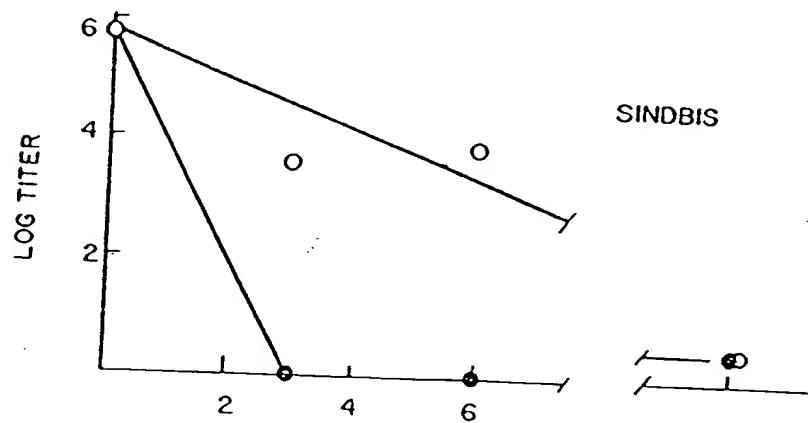
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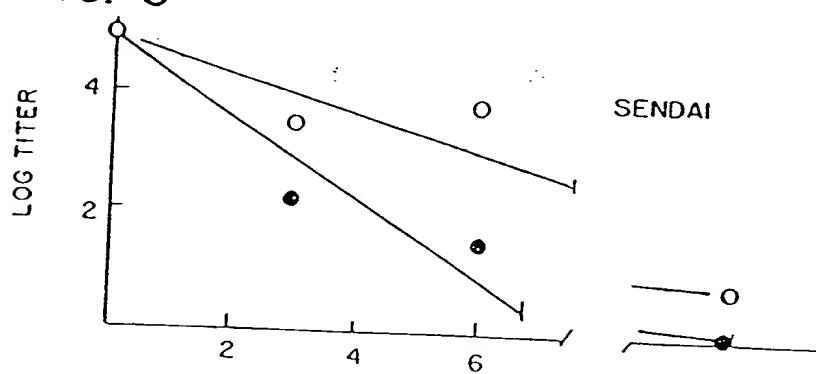
**FIG. 1** COMPARISON BETWEEN 20% ETHER/1% TWEEN 80 (O) AND 0.1% TNBP/1% TWEEN 80 (●)



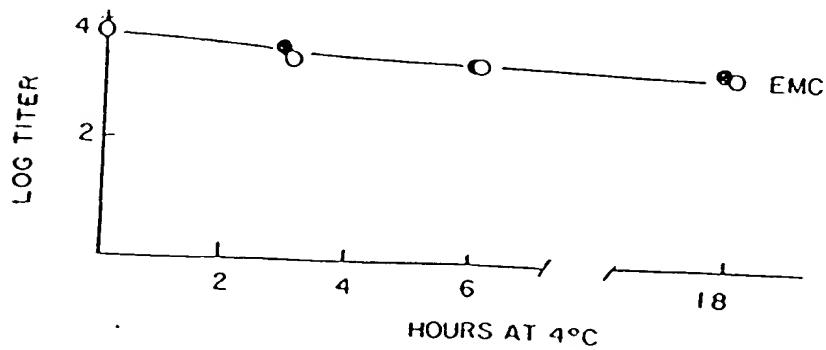
**FIG. 2**



**FIG. 3**



**FIG. 4**





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FIG. 5

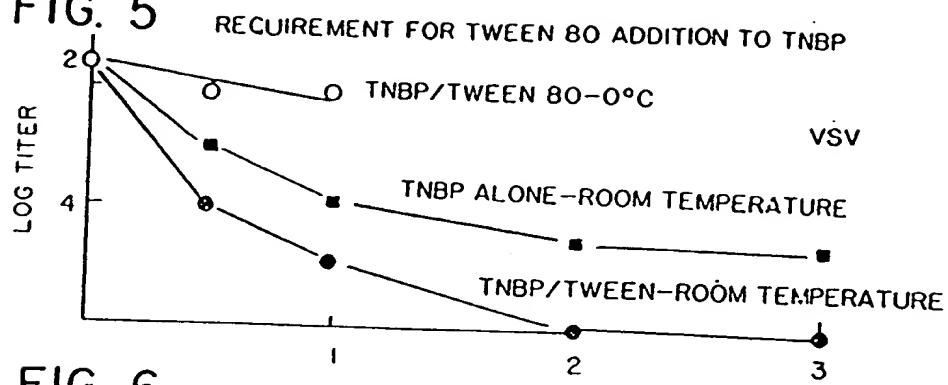


FIG. 6

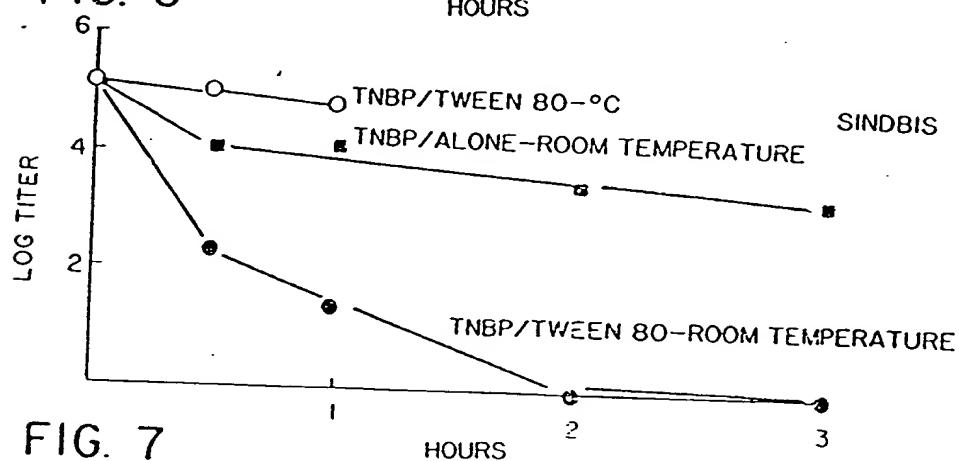


FIG. 7

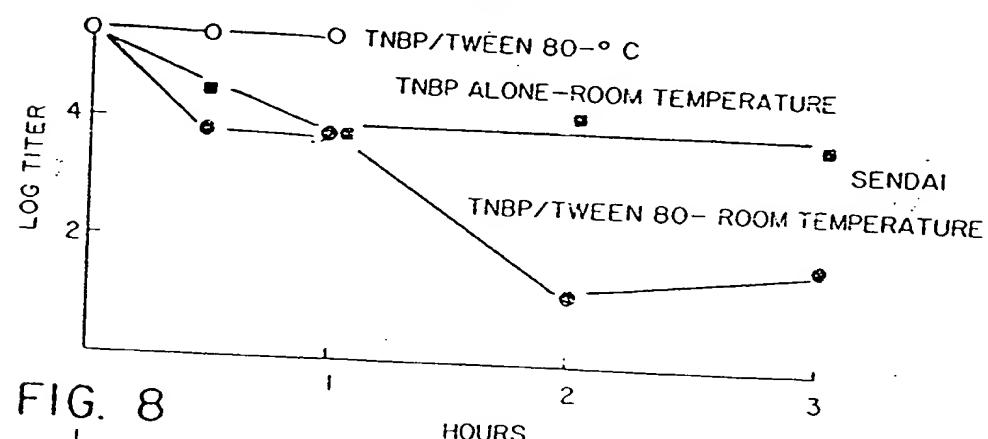


FIG. 8

